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Effect of *Vicia sativa* L. allelochemicals on *Lens culinaris* L. growth and molecular docking against tryptophane synthase β subunit of *Pyrococcus furiosus*

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Vicia sativa is a common weed that grows luxuriously with the lentil (*Lens culinaris* L.) crop. According to literature studies, the plant can produce a negative allelopathic effect on lentil crops. Because both of these plants have the same morphology, and removal through manually picking of the weed is very difficult. The present investigation was framed to identify the allelopathic effect of *V. sativa* by growing lentil plants under the different dilutions of its whole plant extract. We analyzed growth on the 5th and 20th day after seed germination. We estimated the total carbohydrate, nitrogen, and proline contents to assess the potential impact of weed on photosynthesis and respiration. All concentrations of the extract strongly inhibited the growth of the test plant, resulting in a reduction of 3.0 and 2.7 cm in shoot and root length, respectively. The carbohydrate, and nitrogen accumulations decreased in a similar way as compared to its control. The steady increase in free proline contents indicates that whole plant extracts have a negative allelopathic effect. LC/MS analysis of extract revealed presence of various allelochemicals, including luteolin, naringenin, ferulic acid, 3,4-dihydrocinnamic acid, vanillic acid, 3,4-dihydroxyactophenon, succinic acid, and benzaldehyde, among others. These allelochemicals were docked by the tryptophan synthase beta-subunit from *Pyrococcus furiosus* that stimulate tryptophane biosynthesis and auxin production and disturb the phytohormone balance in test plants. Luteolin and naringenin when docked with the target protein (PDB ID 5dw3), show high binding affinity (-9.2 kcal/mol) as compared to their natural ligands.

Keywords: Lentil, Extracts, Allelopathy, Weeds, Carbohydrates, Nitrogen

Weeds are unwanted plants that show phytotoxicity with the crop plants growing nearby. Phytotoxicity refers to any adverse effects on plant growth, physiology, or metabolism. Weeds compete with crop plants for growth, nutrition, and space and generally cause severe phytotoxicity in the form of altered plant metabolism, growth inhibition, etc., by releasing their allelochemicals through leaching. The effect of allelochemicals on the growth and development of other plants is termed allelopathy, and this can be beneficial or may be harmful to the growth of surrounding plants depending upon the condition of the weed and crop plants[1].

Vicia sativa L. is a common annual scrambling and climbing plant belonging to the family Leguminosae. It is moderately tolerant of cold and grows in mild winters[2]. The plant has a highly branched tap root that goes deeper into the soil. The leaves are compound and arranged oppositely on the stem, which terminates into a branched tendril for climbing. The stem is a little hairy and hollow. Flowers are zygomorphic and born in the axil of leaves, and the fruit is a legume pod and contains 4-12 seeds[3]. Since it is a leguminous weed, controlling it in leguminous crops, particularly lentils, can be challenging. Its size mimics that of a lentil seed, making it very difficult to separate[4]. Manual hand picking is sometimes impossible due to the same morphological features. It was estimated that *V. sativa* reduced the yield of the lentil crop by about 20-30%[5]. The plant contains tannins, which decrease its nutritional value and are present as a leucocyanidin equivalent[6]. It also contains a large number of phenolic compounds, such as apigenin, kaempferol, and quercetin, as well as the caffeic, p-coumaric, ferulic, and sinapic acids, chlorogenic acid, procyanidins, gallocatechin, secoisolariciresinol, epigallocatechin gallate, 3′,4′, 7-trihydroxy isoflavone, ononin, isoliquiritigenin, daidzein, glycitein, genistein, daidzin, glycitin, rutin, isoquercetin, hesperidin, and qiercitrin[7,8]. *V. sativa grows in lentils and has negative allelopathic effects.* Traditionally grown in rotation with wheat and rice, lentil (*Lens culinaris* L.) serves as a pulse crop. It has been cultivated in India since very ancient times as a

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pulse crop, and the average yield is 744 kg/ha[9]. In India, lentil production was 1.28 million tons, with an export value of about US$2.80 billion (IIPR ICAR)[10]. It is an important pulse crop as it adds nitrogen to the soil and provides food and nutritional security to the vegan and poor sections of society[11]. The average productivity of the pulses has fallen short due to the increasing demand. To overcome the problem, India imports a large quantity of pulses, and lentils are one of them. Without chemical fertilizers, there is a need to increase productivity and the area of cultivation. Weed is another a major problem that reduces crop productivity, particularly in *V. sativa*.

We conducted the current investigation to examine the impact of *V. sativa*'s allelochemicals on the growth of lentil plants. We are working on *in silico* molecular docking of the allelochemicals against the target protein of the test plant to understand their mechanism of action. The aforementioned study will aid in comprehending the interaction between crops and weeds, as well as identifying potential solutions for sustainable agriculture.

Materials and Methods

Growth analysis

Growth of Lentil was measured in presence of the extract of *V. sativa*. The whole plant was taken from the field and ground after thorough washing. About 1 gram of plant material (whole plant) was ground with 10 mL of distilled water. The solution was centrifuged and the supernatant was collected. The aqueous extract was used to make different dilutions of 5, 10, 25, 50 and 100% with water (v/v). The overnight water-soaked seeds were placed at equal distances in pre-sterilized petri plates. The seeds were irrigated with distilled water as control and in the rest of the Petri plates, different dilutions of extracts were given respectively and accordingly. The plates were incubated at 25 ± 2°C at room temperature and the growth were observed at regular intervals. The shoot-root length was measured with the scales, and the fresh weight and the dry weight were taken using the digital weighing balance.

Plant extract preparation for LCMS analysis

V. sativa plants collected from the field, washed and shade dried at room temperature, and then ground to make a coarse powder. It was then packed in a Soxhlet apparatus (5-40 L) in the form of a thimble. The extraction was done with absolute methanol in Soxhlet for 24-30 h. The extract was dried under reduced pressure to obtain a sticky brown mass, which was again dissolved in methanol and filtered through the Whatman No. 44 filter paper. The extract was concentrated in a rotatory evaporator [Span Automation (SARET43)] at 45^{o}C. The dried methanolic extract was used in different dilutions for further assessing the inhibition of plant growth respectively. The methanolic extract of *V. sativa* was subjected to LCMS analysis on a Waters UPLC/MS mass spectrometer model ACQ-TQD #QBB1152. The ACCUCORE C18, 150 × 2.1, 2.6 mm column was used for separation.

Biochemical analysis

Total nitrogen content

Total nitrogen was estimated in seedling plants of lentil in which all nitrogen present in dry matter was converted into ammonium sulfate through concentrated sulfuric acid. This ammonium sulfate gives a red colour with Nessler's reagent, which was read at 445 nm on a Shimadzu#1800 spectrophotometer. Nitrogen was estimated with the help of a standard curve made by taking different concentrations of standard ammonium sulfate and expressed as mg g-1 dry weight.

Total carbohydrate estimation

For the estimation of total carbohydrate, the dried organic matter was reacted with 2.5 N HCl in a water bath for 3 h to convert carbohydrates into 5-hydroxy methyl furfurals. This when treated with Anthrone reagent gives a green colour. The carbohydrate was estimated by the standard curve prepared by different concentrations of glucose and expressed as mg g-1 dry weight.

Total proline content

About 0.5 g of plant material was homogenized in 10 mL of 3% aqueous sulphosalicylic acid and the extract was filtered through Whatman No. 2 filter paper. About 2 mL of filtrate was taken in a test tube. After that 2 mL of glacial acetic acid and 2 mL acid ninhydrin was added. The mixture was heated in the boiling water bath for 1 h. The reaction was terminated by placing the tube in an ice bath. In the reaction mixture, 4 mL of toluene was added and the mixture was stirred well for 20-30 s. The toluene layer was separated and allowed to attain room temperature. The red colour intensity was measured at 520 nm. For the preparation of the standard curve, a series of standards with pure proline was run similarly.

In silico molecular docking of allelochemicals

Molecular docking-based virtual screening was done to explore the growth inhibitory nature of allelochemicals in the binding pocket of target protein, phytohormone stimulators of tryptophane synthase by using Auto dock vina module of PyRx 0.8 tool. 3D structures of the selected allelochemicals in SDF were downloaded from Pubchem database PubChem (nih.gov). PDB file of the target protein, *Pyrococcus furiosus* tryptophane synthase *ß* subunit 1 (PDB ID 5dw3) was downloaded from RCSB protein data bank (www.rcsb.org) which contain natural ligand (L-tryptophane) co-crystallized with it. Preparation of proteins for molecular docking was performed by using Biovia Discovery studio 2021 and UCSF Chimera 1.16 softwares. With the help of Biovia Discovery studio water molecules and ligands are deleted. Since natural ligand of the target protein is bound to the active site present in Chain A of it, so other chains of the protein were deleted and Chain A was saved in PDB format. Further preparation of protein was performed by dock prep tool of Chimera with default settings where non-complexed ions were deleted, hydrogens and Gasteiger charges were added. The prepared protein was saved in PDB format. Energy minimization of the protein structures was then performed by converting the structure to pdbqt format in the PyRx program, which was used for further molecular docking studies.

Statistical analysis

Data were analyzed using Systat V13.2 software (Systat Software Inc.). Values are presented as means of ten replicates, and the presented means were separated using Duncan's Multiple Range Test (DMRT) at $P \leq 0.05$.

Results

Assessment of the growth and dry weight of lentils

We set up the experiment to expose the lentil seed and seedlings to different concentrations of *V. sativa* whole plant extract. We watched the lentils grow and saw that the root/shoot length and dry weight of the seedlings were significantly ($P<0.05$) lower than the control group. This growth drop was dependent on the extract concentration. We recorded the maximum reduction in root and shoot growth at 100% extract concentration compared to the control, which was 3.0 and 2.7 cm, respectively. In contrast, a severe decline in dry weight occurred at 25-50 (5.7 g) at 100% extract concentration (Fig. 1A & 1B). We noted that the methanolic extract exhibits inhibitory activity.

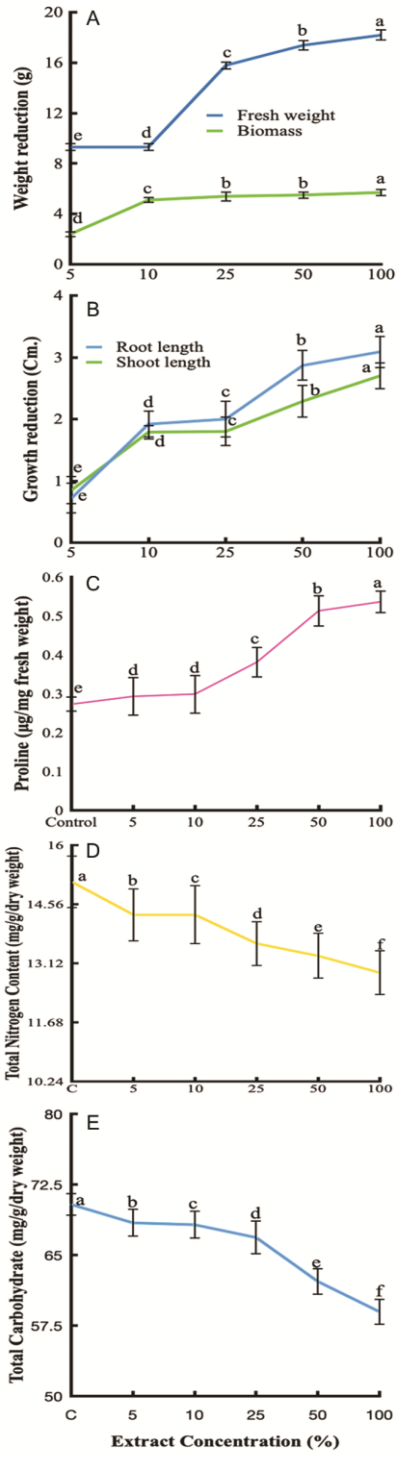

Fig. 1 — Growth of Lentil seedlings under different concentrations of *V. sativa* extract. (A) Fresh and dry weight reduction as compared to control; (B) Growth reduction in root and shoot length; (C) Total proline contents (D & E) Total Nitrogen and Carbohydrate contents. [Values not followed by same letter are significantly different at $P \leq 0.05$. ANOVA with Duncan Multiple Range Test (DMRT); C = Control grown in distilled water without extract]

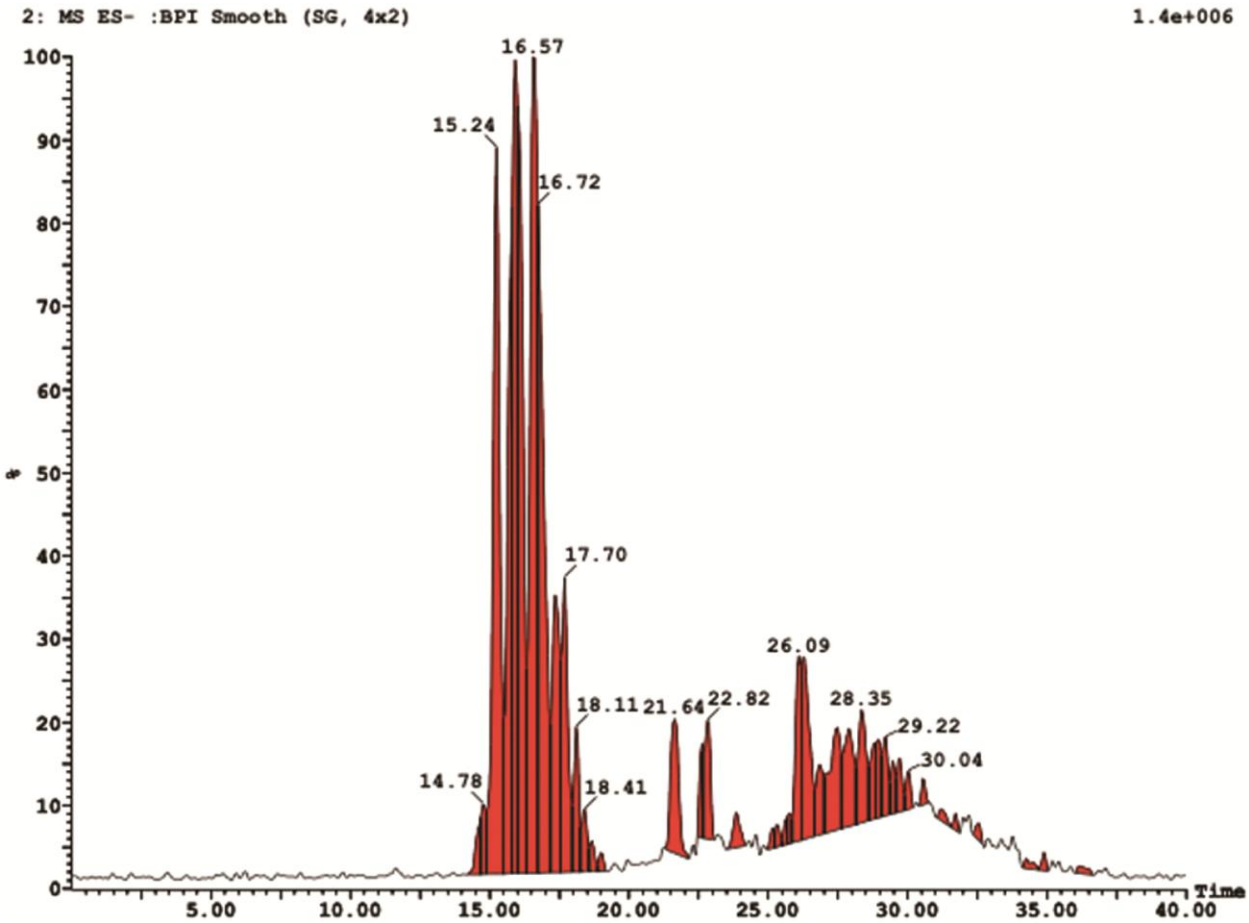

Fig. 2 — Chromatogram of the LCMS of methanolic extract of the *V. sativa* plant

Biochemical analysis during growth stages revealed that there was a reduction in the tested organic compounds, carbohydrates, and total nitrogen accumulation. Total carbohydrate was significantly (P<0.05) decreased in the lentil seedlings with increased extract concentrations. The 100% extract concentration showed a significant reduction of 58.8 mg/g dry weight compared to the distilled water-grown control. Total nitrogen content was also significantly (P<0.05) reduced with the increasing extract concentration. This shows that the extract's effect has reduced the nitrogen use efficiency (NUE) of the lentil seedling plants (Fig. 1C, 1D & 1E).

We also studied the proline accumulation in extract-treated lentil plants to analyze the extract-induced stress level. The proline accumulation significantly increased from 5 to 100% extract concentration. This indicates the seedlings were under stress but sustained.

LCMS analysis of the methanolic extract of *V. sativa*

The LCMS analysis of *V. sativa* methanolic extract revealed the presence of 10 allelochemicals with varying peaks. The plants leached out various classes of organic compounds to suppress the growth and yield of lentil plants. There are a number of these allelochemicals, such as vanillic acid, ferulic acid, 2,6-dihydrobezoic acid, succinic acid, 3,4-dihydrocinnamic acid, benzaldehyde, luteolin, and naringenin. Fig. 2 and Table 1 represent the chromatogram and the RT of the allelochemicals.

Table 1 — List of compounds identified in LCMS analysis of *V. sativa* whole plant extract

Compound Name	Molecular Formula	Molecular weight (g/mol)	Retention Time
3, 4-dihydroxybenzoic acid	$C_7H_6O_4$	154.12	16.63
3, 4-dihydroxyacetophenon	$C_8H_8O_3$	152.15	15.91
Vanillic acid	$C_8H_8O_4$	168.14	14.70
Ferulic Acid	$C_{10}H_{10}O_4$	194.18	18.32
2,6-dihydroxybezoic acid	$C_7H_6O_4$	154.12	17.35
Succinic acid	$C_4H_6O_4$	118.09	18.12
3,4-dihydroxycinnamic acid	$C_9H_8O_4$	180.16	22.22
Benzaldehyde	C_6H_5CHO	106.12	14.78
Luteolin	$C_{15}H_{10}O_6$	286.24	6.24
Naringenin	$C_{27}H_{32}O_{14}$	272.25	6.80

Molecular Docking results of allelochemicals

Tryptophane generally synthesizes the phytohormone IAA in plants. The tryptophane synthase *ß* subunit 1 (TSB1) is involved in the synthesis of tryptophane and IAA[12]. Allelochemicals disrupt plant growth regulators and impair plant growth. They stimulate the tryptophane synthase enzyme. Molecular docking was performed *via* the AutoDockVina module of PyRx with prepared Chain A of *Pyrococcus furiosus* tryptophane synthase *ß* subunit 1 (PDB ID 5dw3) as target protein with these allelocompounds as ligands. Following molecular docking, we discovered that luteolin and naringenin exhibited a higher binding affinity (-9.2 kcal/mol) than their natural tryptophane ligand (-7.8 kcal/mol). However, the other allelochemical also showed excellent binding affinity in the range of -7.8 to -4.9 (Table 2 & 3). Our chosen allelochemicals for the present study have a favorable binding interaction with *Pyrococcus furiosus* tryptophane synthase *ß* subunit 1. The binding affinity of luteolin and naringenin (-9.2 kcal/mol) followed by ferulic acid (-7.3 kcal/mol), 3,4-dihydrocinnamic acid (-6.8 kcal/mol), vanillic acid (-6.4 kcal/mol), and 3,4-dihydroxyactophenon (-6.3 kcal/mol), succinic acid, and benzaldehyde (5.2). These results indicate that the above mentioned allelochemicals played a significant role in inhibition of protein synthesis and auxin accumulation and thus retarded the overall growth of the plant (Table 2; Fig. 3A & 3B).

Table 2 — List of compounds their binding affinities with the target protein of auxin biosynthesis

Compound Name	Binding Affinity	PubChem ID
3, 4-dihydroxybenzoic acid	-6.1	72
3, 4-dihydroxyacetophenon	-6.3	14530
Vanillic acid	-6.4	8468
Ferulic Acid	-7.3	445858
2,6-dihydroxybezoic acid	-6.4	9338
Succinic acid	-5.2	1110
3,4-dihydroxycinnamic acid	-6.8	57078473
Benzaldehyde	-5.2	240
Luteolin	-9.2	5280445
Naringenin	-9.2	439246
L-Tryptophan	-7.8	6305

Discussion

Allelochemicals secreted from one plant inhibit the growth of other plants growing nearby, and it is a common phenomenon of negative allelopathic interaction. The *L. culinaris* plant grown under *V. sativa* whole plant extract showed significant growth retardation, both morphologically and physiologically. Allelochemicals impede metabolic processes and cell division in the rhizosphere, resulting in a reduction in crop plant root and shoot growth[13]. The present study found that the effects of allelochemical compounds reduced root and shoot growth. Benzoic acid and its derivatives are the most potent allelochemicals secreted by the *V. sativa* plant, and they cause harm to the receptor plant as lentils. Its molecular mechanism is unknown, but it was reported earlier that *p*-hydroxybenzoic acid affects the meristem activity and cell length due to reduced

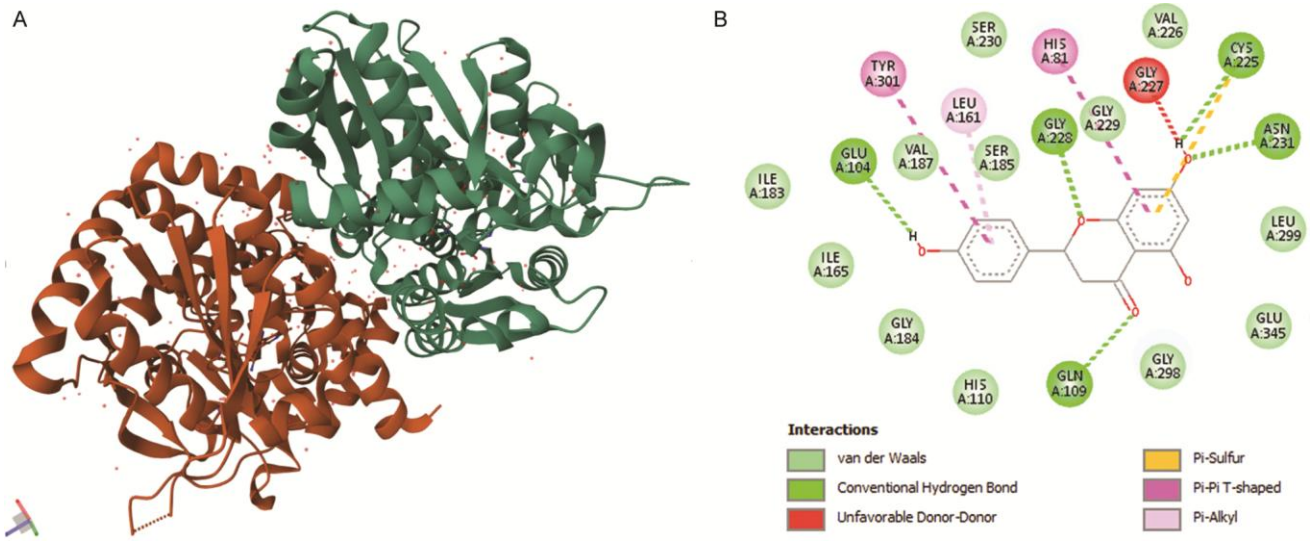

Fig. 3 — (A) 3D structures of Tryptophan Synthase β subunit from *Pyrococcus furiosus* with product L-tryptophan non-covalently bound in the active site (adapted from PDB id 5dw3; Buller *et al*., 2015); (B) Molecular interaction of the allelo-compound luteolin with the TSB1 tryptophane receptor.

antioxidant activity in the root tips of cucumber[14]. Allelochemicals can inhibit mitosis and cause mitotic anomalies by damaging chromatin organization and the mitotic spindle, as reported in earlier studies[15,16]. Growth reduction in lentils significantly impacted biomass accumulation compared to the control group. Allelochemicals, which are supposed to increase soil organic carbon, total nitrogen, microbial carbon utilization, and the microflora, caused a decrease in biomass accumulation and growth reduction. Allelochemicals can alter the soil's microbial flora, disrupting the regular decomposition process[17,18]. Certain phenolic acids, such as quinolinic acid and other allelochemicals, increase in plants subjected to mineral deficiencies, water shortages, abnormal radiation, temperature extremes, and herbicides. Benzoic acids, the most potent allelochemical, significantly reduce the mineral content of the affected plant[19]. The benzoic acid and derivative allelochemicals somewhat inhibit the root growth of lentils, as they are reported to be involved in the inhibition of primary root elongation in *Arabidopsis*, a process that also affects the auxin signaling process. It stimulates the increased expression of the auxin biosynthesis gene and its polar transport[20]. The auxin synthesis takes place in plants in two pathways: the tryptophane dependent pathway and the tryptophane independent pathway. Benzoic acid binds to receptors of the tryptophane biosynthetic pathway, enhancing auxin accumulation and inhibiting root growth. Tryptophan (*Trp*) synthase *ß* subunit 1 (TSB1) is essential for tryptophane synthesis in plants; thus, it affects protein biosynthesis, auxin accumulation, and plant growth[21,22]. Allelochemicals may cause phytohormone imbalance and slow the overall growth of the test plant. This may be the possible reason of the reduced growth under the *V. sativa* whole plant extract.

Among all the allelochemicals we tasted, luteolin and naringenin had the highest binding affinity with TSB1. Chemically, it is 3′,4′,5,7-tetrahydroxyflavone, which is present in most plants and has multifunctional biological activity[23]. It has anti-inflammatory antioxidant activity used as a chemopreventive drug that inhibits tumor cell proliferation and metastasis[24,25]. Luteolin is believed to disrupt the auxin level in lentil plants, thereby either directly or indirectly suppressing growth. It is also thought that auxin helps reorganize the cytoskeleton in the pericycle and endodermis during the early stages of lateral root organogenesis. In root epidermal and hypocotyl cells, auxin rapidly triggered microtubule rearrangement for growth[26]. The above results indicate that luteolin is also indirectly involved in growth inhibition in plants.

Conclusions

The current study demonstrated the potential of allelochemicals produced by weed plants. Our results showed that there is strong allelopathic interaction between *V. sativa* and *L. culinaris* L**.** The LCMS analysis of *V. sativa* methanolic extract revealed that there are some potent allelochemicals that suppress the growth of lentil. Weed infestation, which mimics the morphological characteristics of lentils, poses a significant problem in the production process. The weed plant, like *V. sativa*, interferes with phytohormone synthesis and disturbs their homeostatic balance, inhibiting crop growth and reducing yield. Bio-herbicides can effectively eradicate weeds. In sustainable agriculture, the development of allelochemical-based bio-herbicides can be useful. The allelochemicals and their likely impact on plant physiology may further encourage the development of effective new eco-friendly bio-herbicides.

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Conflict of interest

The authors declare that no conflict of interest

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